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Ovine Skeletal Muscle Multicatalytic Proteinase Complex (Proteasome): Purification, Characterization, and Comparison of Its Effects on Myofibrils with μ-Calpains[1,2]

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ABSTRACT: The latent form of multicatalytic proteinase complex (MCP) was purified to homogeneity from ovine skeletal muscle. The MCP ran as a single band (M_r 600,000) on nondenaturing polyacrylamide gel (PAGE) and dissociated to a number of subunits (M_r 21,000 to 31,000) under denaturing and reducing conditions (SDS-PAGE). The proteinase complex was activated reversibly by heating at 60°C and in the presence of SDS. Maximum activation (18-fold) was observed after 2 min at 60°C and there was rapid inactivation beyond 2 min. Maximum proteolytic activity (12.8-fold) occurred in the presence of .25 m*M* SDS and diminished rapidly at higher SDS concentrations. The MCP was maximally active at pH 7.5 to 8.0 and 45°C using radiolabeled α-casein. These and other results (e.g., proteinase inhibitor profiling) indicate that ovine skeletal muscle does indeed contain MCP and that its biochemical properties are the same as MCP isolated from other sources. By using [^{14}C]-casein as a substrate, the specific activities (milligrams of protein degraded/milligrams of proteinase) for μ-, m-calpain, and MCP were 44.0, 59.7, and 2.0, respectively. Purified ovine myofibrils were incubated with μ-calpain or MCP. Classical effects of calpains, which include degradation of Z-disks, titin, desmin, troponin-T, and troponin-I and removal of α-actinin, were observed. However, only troponin-C and myosin light chains-2 and -3 were degraded by MCP. Morphologically, MCP had no detectable effect on myofibrils. Results suggest that MCP is not involved in the initial steps of myofibril disassembly. However, its involvement in the degradation of myofilaments remains to be determined.

Key Words: Skeletal Muscle, Proteinases, Multicatalytic Proteinase Complex, Calpain

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Introduction

The multicatalytic proteinase complex (**MCP**), (M_r 650 to 700 kDa) a nonlysosomal proteinase, was first isolated from bovine pituitaries (Orlowski and Wilk, 1981) and subsequently purified from a number of different mammalian tissues. A similar proteinase has also been found in fish, lobster, and yeast (reviewed by Rivett, 1989; Orlowski, 1990). The proteinase complex appears as a series of low molecular weight, nonidentical subunits on SDS-PAGE. The proteinase was first recognized as "multicatalytic" (Wilk and Orlowski, 1980, 1983) because multiple synthetic substrates are degraded. The MCP is usually extracted in a latent

[1]Mention of a trade name, proprietary product, or specific equipment is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

[2]I am grateful to A. Kruger for electron microscopy analysis; P. Bures for photography; S. Hauver, G. Whipple, and J. R. Arbona for their assistance in the execution of these experiments; and to M. Bierman for her secretarial assistance. I am also grateful to G. N. DeMartino for his generous gift of antibody against multicatalytic proteinase complex.

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state and selectively activated by heat (Tanaka et al., 1986a; Mykles, 1989a,b), polylysine (Tanaka et al., 1986a; Mellgren, 1990), low concentrations of SDS (Wilk and Orlowski, 1983; Dahlmann et al., 1985b; McGuire and DeMartino, 1986; Tanaka et al., 1986a; McGuire et al., 1989; Mykles, 1989a), and dialysis against water (McGuire et al., 1989). Electron microscopy studies of MCP from different species have indicated similar cylinder-shape particles with dimensions of approximately 15 nm × 11 nm (Kopp et al., 1986; Tanaka et al., 1986b; Mykles, 1989a).

This proteinase has been referred to by a variety of names, but now there is general agreement to call it MCP (Rivett, 1989). Though its function is not known, it is proposed that MCP plays a significant role in nonlysosomal protein turnover (Rivett, 1989; Orlowski, 1990), and it has been hypothesized that it could be involved in the degradation of muscle proteins (Goll et al., 1989). This proteinase complex has also been implicated in the ATP/ubiquitin-dependent and ATP-dependent, ubiquitin-independent pathways of intracellular protein degradation (Ma et al., 1992).

The objectives of the experiments described here were to determine the existence of MCP in ovine skeletal muscle and to characterize its biochemical properties and effects on myofibrils.

Materials and Methods

Materials. All chromatographic materials, with the exception of hydroxyapatite, which was purchased from Supelco (Bellefonte, PA), were from Pharmacia-LKB (Piscataway, NJ). Immobilon-p was from Millipore (Bedford, MA). All electrophoretic grade chemicals were obtained from Bio-Rad Laboratories (Richmond, CA). Leupeptin and E-64 were from Peptide International (Louisville, KY); PMSF and Aprotinin were from Boehringer Mannheim (Indianapolis, IN). All other chemicals, which were analytical grade or purer, were obtained from Sigma Chemical (St. Louis, MO).

Purification of Ovine Multicatalytic Proteinase Complex. The latent form of MCP was purified from ovine skeletal muscle by slight modifications of existing procedures (McGuire and DeMartino, 1986; McGuire et al., 1989; Mykles, 1989a; Mason, 1990). Briefly, 3.9 kg of ovine skeletal muscle obtained within 30 min of slaughter was homogenized in 2.5 volumes of 50 m*M* Tris-HCl, pH 8.3 that contained 10 m*M* EDTA and 10 m*M* β-mercaptoethanol. After centrifugation, the homogenate initially fractionated between 0 to 45% and subsequently 45 to 65% ammonium sulfate. The 0 to 45% fraction was used to purify μ- and m-calpain as described previously (Koohmaraie, 1992a). The 45 to 65% fraction was dialyzed against buffer A (40 m*M* Tris-HCl, pH 7.5 that contained .5 m*M* EDTA, and 10 m*M* β-mercaptoethanol) that contained 100 m*M* NaCl. After centrifugation, the dialyzed fraction was loaded onto a 5-cm × 25-cm column of DEAE-Sephacel equilibrated with the same buffer. Unbound proteins were removed by washing the column with the same buffer until outflow absorbance at 278 nm was < .1. Bound proteins were eluted with a linear gradient of NaCl from 100 to 300 m*M*, fractions with MCP activity were pooled, concentrated (YM-100 membrane; from Amicon, Beverly, MA), adjusted to 500 m*M* NaCl, and loaded onto a 2.6-cm × 40-cm column of Phenyl-Sepharose. Under these conditions, MCP does not bind to the resin, although most of the impurities are retained. Active fractions were pooled, salted out between 0 to 65% ammonium sulfate, and loaded onto a 1.6-cm × 60-cm column of Superdex 200 (HiLoad; FPLC) in three runs. Buffer A was used during all chromatography steps and, in the case of gel filtration steps, it also included 1 m*M* NaN_3. Fractions from the Superdex 200 column that contained MCP activity were pooled and used for characterization of the MCP. Purification was monitored throughout by 1) heating 100-μL aliquots of each fraction and 2) SDS-PAGE analysis of all fractions from all chromatographic steps, using the characteristic MCP subunit composition for identification.

Assay for Multicatalytic Proteinase Complex Activity. Caseinolytic activity was determined using [^{14}C]methylcasein (4,088 cpm/μg; 10 μg) as the substrate. The MCP was routinely heated in 100-μL aliquots at 60° C for 2 min and chilled on ice. The reactions (100 μL) consisted of 65 μL of buffer (buffer B; 50 m*M* Tris-HCl, pH 7.75 that contained 1 m*M* NaN_3), 25 μL of heated and chilled MCP, and 10 μL of radiolabeled casein. After 60 min at 45° C (before determination of optimum temperature, incubations were done at 37° C), 100 μL of 10 mg/mL BSA (carrier) was added and proteins precipitated with equal volumes of 10% cold trichloroacetic acid (TCA) and centrifugation at 8,800 × *g* for 15 min. A uniform aliquot of TCA-soluble radioactivity was determined with a Packard model 460 liquid scintillation counter (Packard Instrument, Meriden, CT).

Heat Activation of Multicatalytic Proteinase Complex. Pooled fractions from Superdex 200 columns were heated in 100-μL aliquots (45.9 μg of MCP) at 60° C from 0 to 10 min. After chilling on ice for 10 min, the heated fractions were centrifuged for 6 min at 8,800 × *g* at 4° C and 25-μL aliquots (i.e., 11.5 μg of protein) assayed as described above.

Determination of Optimum pH. Pooled fractions from Superdex 200 columns were heated at 60°C for 2 min in 100-µL aliquots, chilled on ice, and centrifuged. Caseinolytic activity was determined over a pH range of 5.5 to 9.0. For pH 5.5 to 7.0, the buffer used was Tris-malate (conductivity of all buffers between 5.5 to 7.0 was 1.5 µS) and for pH 7.5 to 9.0 Tris-HCl (conductivity of buffers was 1.9, 1.6, 1.2, and 1.0 µS for pH 7.5, 8.0, 8.5, and 9.0, respectively). Assays were done as described above. Buffer conductivity was determined with a Radiometer conductivity meter (Cleveland, OH).

Determination of Optimum Temperature. Pooled fractions from Superdex 200 columns were heated in 100-µL aliquots for 2 min at 60°C, chilled on ice, centrifuged, and assayed (25 µL of heated fractions [i.e., 11.5 µg of protein]) for casein hydrolysis as described above but using different temperatures from 5 to 60°C.

Activation with Sodium Dodecyl Sulfate. Twenty-five microliters of MCP (11.5 µg) from pooled fractions off Superdex 200 columns plus 65 µL of buffer B were incubated for 10 min at 45°C in the presence of increasing concentrations of SDS from 0 to 4 m*M* SDS by adding sufficient volume of 17.3 m*M* SDS to give the desired final concentration. Afterwards, 10 µL of radiolabeled casein was added and caseinolytic activity was determined as described above.

Removal of Sodium Dodecyl Sulfate. To determine the irreversibility of SDS effect, aliquots of MCP preincubated with .25 m*M* SDS were applied to prepacked Extracti-gel D columns (Pierce Chemical, Rockford, IL) to remove SDS. As a control, aliquots of MCP not incubated with SDS were also chromatographed. After chromatography, preincubated samples, preincubated samples chromatographed on Extracti-gel columns, and unincubated samples chromatographed on Extracti-gel columns were assayed as described above.

Effects of Ionic Strength. Aliquots of MCP from Superdex 200 column were heated at 60°C for 2 min, chilled, centrifuged, and assayed in the presence of increasing concentrations (0 to 1,000 m*M*) of NaCl. Sufficient volume from 3.0 *M* NaCl was added to give the desired final concentration.

Effects of Different Compounds on Caseinolytic Activity of Multicatalytic Proteinase Complex. Effects of different compounds (Table 1) on the caseinolytic activity of MCP were determined following the same protocol as described for ionic strength effects. All compounds were made as stock solutions and sufficient volume of stock solution was added to give the desired final concentration.

Assay of µ- and m-Calpain. The caseinolytic activity of µ- and m-calpain were determined using radiolabeled casein as substrate. The reaction consisted of 45 µL of buffer (Tris-acetate pH 7.5 containing 10 m*M* β-mercaptoethanol, 1 m*M* NaN_3, and 300 µ*M* [µ-calpain], or 5 m*M* [m-calpain] $CaCl_2$), 2.95 µg of µ-calpain, or 2.58 µg of m-calpain and 50 µL of radiolabeled casein (289 µg, 523 cpm/µg). Parallel control reaction contained 10 m*M* EDTA instead of $CaCl_2$, accompanied the reaction. After 60 min at 25°C, reactions were stopped by sequential addition of 100 µL of BSA (10 mg/mL) and 200 µL of 10% TCA and radioactive content of the TCA-soluble fraction determined as described for MCP.

Table 1. Effect of various compounds on the caseinolytic activity of ovine skeletal muscle multicatalytic proteinase complex[a]

Compound	Final concentration	% of control
Control	—	100.0
Iodoacetate	1.0 m*M*	94.4
Leupeptin	.67 m*M*	93.0
E-64[b]	.75 m*M*	96.4
Hemin	.1 m*M*	0
PMSF[c]	1.0 m*M*	91.7
PMSF	5.0 m*M*	92.6
PMSF	10.0 m*M*	82.3
Aprotinin	1.0 mg/mL	69.0
Ovomuciod	1.0 mg/mL	79.2
Mercaptoethanol	5.0 m*M*	100.0
EDTA	1.0 m*M*	100.0
$CaCl_2$	1.0 m*M*	100.0
$MgCl_2$	1.0 m*M*	101.5
$NiCl_2$	1.0 m*M*	79.0
$CoCl_2$	1.0 m*M*	52.8
$ZnCl_2$	1.0 m*M*	5.3
$CuSO_4$	1.0 m*M*	1.5
$HgCl_2$	1.0 m*M*	.8

[a]Enzyme solution of .459 mg/mL was heated in 100-µL aliquots after chilling on ice and centrifugation, sufficient enzyme (in 100-µL aliquots) was heat activated for one replication of the above experiments. Then 25 µL (11.5 µg) was used to determine compound effect. The MCP and the respective compounds were incubated at 21°C for 10 min, before substrate was added and proteolytic activity was determined as described in the Materials and Methods section. These experiments were repeated three times in triplicate. The CV was < 10%.

[b]*t*-epoxysuccinyl-L-leucylamido-(4- guanidino)-butane.

[c]Phenylmethylsulfonyl fluoride.

Incubation of Myofibrils with Multicatalytic Proteinase Complex and µ-Calpain. Myofibrils purified from ovine skeletal muscle obtained within 30 min of slaughter (Goll et al., 1974) were incubated with MCP at a ratio of 2:1 (myofibrils:MPC). The reaction consisted of 58.8 µL (1,000 µg) of purified myofibrils, 359 µL of 50 m*M* Tris-HCl, pH 7.75, 7.2 µL of 17.3 m*M* SDS, and 75 µL of MCP (500 µg). All components were incubated at 45°C for 10 min to activate MCP, myofibrils were added and the incubation continued for another 4 h. To prevent sedimentation of myofibrils, the tubes were gently vortexed every 15 min. Control tubes (no MCP) were treated similarly. Myofibrils were incubated with µ-calpain at 25°C in a ratio of 10:1 (myofibrils:

μ-calpain) for 4 h. The reaction consisted of 58.8 μL myofibrils (1,000 μg), 424 μL of 50 m*M* Tris-HCl, pH 7.5 that contained 10 m*M* β-mercaptoethanol, 1 m*M* NaN_3, and 3 m*M* $CaCl_2$, or 10 m*M* EDTA. Tubes were vortexed gently every 15 min. At the completion of the incubation period (μ-calpain reaction stopped by addition of EDTA to 10 m*M*), myofibrils were sedimented at 1,000 × *g* and washed once with buffer. Aliquots were removed for SDS-PAGE (Koohmaraie, 1990) and for phase and transmission electron microscopy (Koohmaraie et al., 1986).

Radiolabeling of Casein. Alpha-casein was radiolabeled with [^{14}C]-formaldehyde as described by Dottavio-Martin and Ravel (1978).

Measurement of Protein. Protein concentrations were determined by the method of Bradford (1976) using premixed reagents purchased from Bio-Rad. Myofibril protein concentration was determined using the Biuret procedure (Gornall et al., 1949) with BSA as the standard.

Denaturing (SDS-PAGE) and Nondenaturing Polyacrylamide Gel Electrophoresis (PAGE). The SDS-PAGE was performed on 12.5% gels as described by Laemmli (1970). Myofibrillar proteins were separated with a linear gradient of acrylamide from 7.5 to 15% as described by Koohmaraie (1990). The PAGE was conducted on 5% gels according to the procedures described in the Hoeffer Scientific catalog (1992).

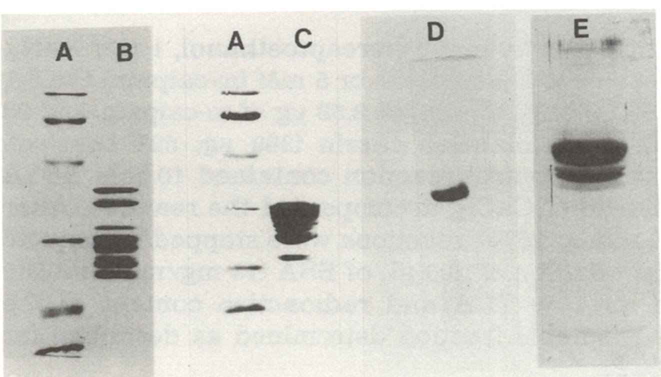

Figure 1. The SDS-PAGE (12.5%) analysis of pooled muscle multicatalytic proteinase complex (MCP) fractions (12.3 μg) from Superdex 200 (lane B) and hydroxyapatite (lane C) columns. Nondenaturing polyacrylamide (5%) analysis of MCP (20.6 μg) from hydroxyapatite chromatography (lane D) and Western blot (15% SDS-PAGE) demonstrating cross-reactivity between ovine skeletal muscle MCP and anti-MCP from erythrocytes (lane E). Lane A is the molecular weight standards that consist of rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa).

Sephacryl S-300 Chromatography for M_r Estimation. Gel filtration of pooled MCP from Superdex 200 was carried out on Sephacryl S-300 column (1.6 cm × 60 cm; HiLoad, high resolution; FPLC) equilibrated with buffer A. The column was calibrated with blue dextran (void volume) and molecular weight standards thyroglobulin (M_r = 660,000), ferritin (M_r = 440,000), β-amylase (M_r = 200,000) and bovine serum albumin (M_r = 66,000).

Immunoblotting. To test the cross-reactivity of ovine MCP with antibody raised against human erythrocytes (provided by G. N. DeMartino, University of Texas at Dallas), ovine MCP was transferred to Immobilon-P membrane and probed with erythrocyte anti-MCP (McGuire et al., 1988) as described previously (Koohmaraie, 1992a).

Results

Purification of Ovine Multicatalytic Proteinase Complex

The purification protocol as outlined in the Materials and Methods section resulted in significant purification of ovine skeletal muscle MCP (Figure 1B). The elution pattern of ovine skeletal muscle MCP from anion-exchange resin was essentially the same as those reported for other tissues (Dahlmann et al., 1983, 1985a; McGuire and DeMartino, 1986; Tanaka et al., 1986a; Yamamoto et al., 1986; Zolfaghari et al., 1987). Ovine skeletal muscle MCP eluted as a single major fraction on DEAE-Sephacel between 230 and 280 m*M* NaCl. Chromatography on hydrophobic resin (Phenyl-Sepharose) was very effective in removing the contaminating proteins. However, during Superdex 200 and especially Sephacryl S-300 chromatography (for size estimation), it became apparent that the top two bands (Figure 1B) that copurified with MCP are not part of the enzyme complex. To remove these impurities, pooled fractions from Superdex 200 columns were concentrated and loaded onto Sephacryl S-300. Fractions that contained MCP activity were pooled and loaded onto a 2.5-cm × 20-cm column of hydroxyapatite, which removed the remaining contaminates, leaving only a series of low molecular weight polypeptides that ranged from 21 to 31 kDa (Figure 1C).

Characterization of Multicatalytic Proteinase Complex

Purified ovine skeletal muscle MCP gave a single protein band on nondenaturing PAGE (Figure 1D), which under denaturing and reducing conditions disassociated to a series of low molecular weight subunits ranging from 21 to 31 kDa

(Figure 1C). The appearance of multiple components under SDS-PAGE has been observed previously (reviewed by Rivett, 1989; Orlowski, 1990). These components are distinct subunits of the large complex and products of different genes, rather than the result of autodigestion (Lee et al., 1990; DeMartino et al., 1991). Size estimation on gel filtration indicated that ovine skeletal MCP had an apparent molecular weight of approximately 600,000 (Figure 2).

Like MCP from other mammalian tissues and from fish, lobster, and yeast, ovine skeletal muscle MCP did not exhibit any proteolytic activity without activation. The MCP was activated by heating at 60° C. Maximum stimulation occurred with 2 min at 60° C (18-fold increase) and heating beyond 2 min resulted in rapid loss of caseinolytic activity (Figure 3). However, the heat-activation was a reversible process (Figure 4). When heated MCP that was stored at 4° C for 72 h was reheated (i.e., for 2 min at 60° C), it only had 70.2% of its original activity (Figure 4). The MCP could also be activated by pretreatment with low concentration of SDS. Maximum stimulation occurred in the presence of .25 m*M* SDS (12.8-fold). At concentrations > .25 m*M* SDS, MCP rapidly lost its caseinolytic activity such that at 1.0 m*M* SDS no proteolytic activity could be detected (Figure 5). To determine the reversibility of SDS effect, MCP was first activated in the presence of .25 m*M* SDS and then SDS was removed by chromatography on Extracti-gel D. Removal of SDS decreased proteolytic activity by > 85%, clearly suggesting that SDS activation of ovine MCP is largely reversible (data not shown). These results agree with those of

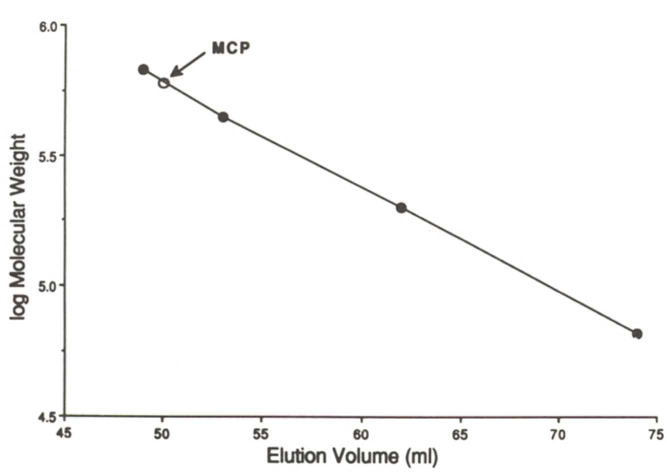

Figure 2. The M_r-estimation of the hydroxyapatite-purified muscle multicatalytic proteinase complex (MCP) on Sephacryl S-300. Arrow indicates the position at which MCP eluted. For details see Materials and Methods section.

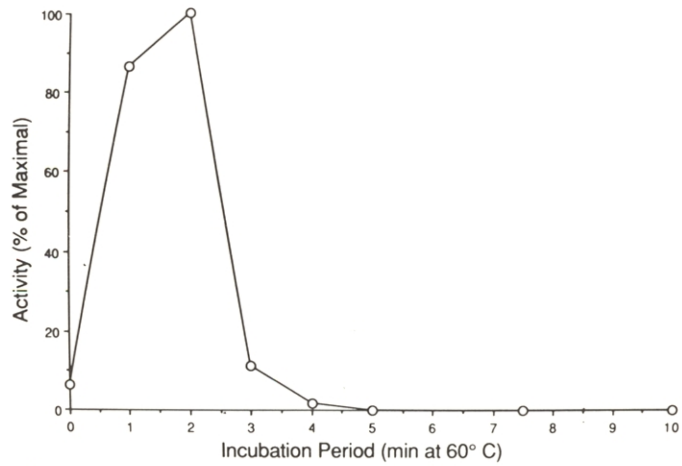

Figure 3. Effect of heating at 60°C on the caseinolytic activity of ovine muscle multicatalytic proteinase complex (MCP). The 100-µL aliquots of an enzyme solution (.459 mg/mL) were heated at 60°C from 0 to 10 min and an aliquot of heated fractions was assayed for MCP as described in the Materials and Methods section. Maximum activity represents an 18-fold increase in activity.

McGuire et al. (1989), who reported that upon removal of SDS, erythrocytes MCP lost 70% of its activity. Orlowski et al. (1991) have recently determined that SDS stimulates proteolytic activity of MCP by inducing conformational changes at a site or sites different from the substrate binding site.

Temperature and pH optimum of ovine skeletal MCP for casein degradation were 45° C and 7.5 to

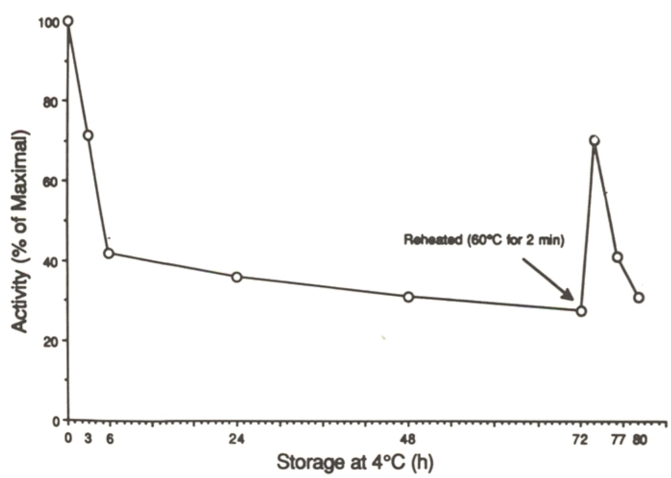

Figure 4. Reversibility of heat activation of ovine muscle multicatalytic proteinase complex. The 100-µL aliquots of an enzyme solution (.459 mg/mL) were heated at 60°C for 2 min and an aliquot of heated fractions was assayed for caseinolytic activity from 0 to 80 h after heating. Maximum activation represents an 18-fold increase in the activity.

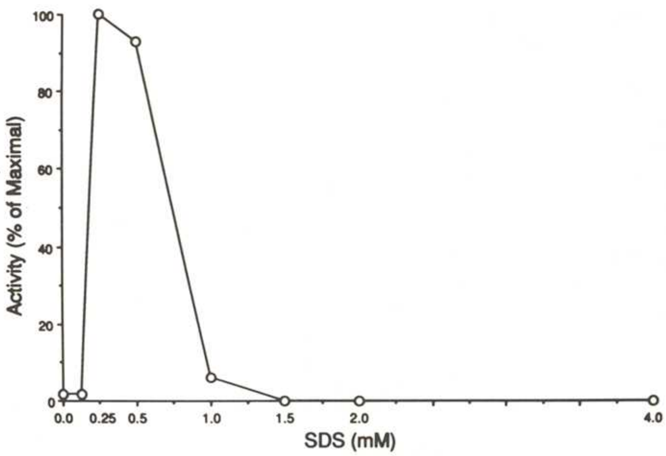

Figure 5. Effect of SDS on the caseinolytic activity of ovine muscle multicatalytic proteinase complex (MCP). Aliquots of MCP (11.5 μg) were preincubated with different concentrations of SDS from 0 to 4 m*M* for 10 min at 45°C and then substrate was added. Proteolytic activity was determined as described in the Materials and Methods section. Maximum activation represents a 12.8-fold increase in the activity.

8.0 (Figures 6 and 7). Activity of MCP is often determined at 37° C, and at least in one laboratory MCP is assayed at room temperature (Mykles, 1989a,b). These results indicate that maximum casein hydrolysis occurs at 45° C, which agrees with the temperature profile reported for MCP (Ishiura and Sugita, 1986). Because of the multicatalytic characteristics of MCP, the pH optima depends on the substrate used, present observations of pH optima for casein hydrolysis (7.5 to 8.0) are similar to previous reports (Tanaka et al., 1986a; Mykles, 1989a,b).

The MCP activity increased with increasing ionic strength up to 100 m*M* NaCl but lost activity at high salt concentrations. At 1,000 m*M* NaCl, MCP caseinolytic activity was 54.7% of that at 100 m*M* NaCl (Figure 8). Similar observations with respect to loss of activity with increased ionic strength have been reported by Mason (1990).

Table 1 indicates the effect of various compounds on the caseinolytic activity of MCP. At high concentrations (10 m*M*), the serine protease inhibitor, phenylmethylsulfonyl fluoride, decreased MCP activity only slightly (17.7%). Aprotinin, another serine proteinase inhibitor, also partially (31%) inhibited MCP activity. Inhibitors of cysteine proteinases (E-64, leupeptin, and iodoacetate) had no effect on the caseinolytic activity of MCP. Reducing agents (e.g., β-mercaptoethanol) did not stimulate MCP activity. The metalloproteinase inhibitor (EDTA) had no effect on MCP activity. Ovine MCP was not effected by $CaCl_2$ or $MgCl_2$. However, it was inhibited by $NiCl_2$ (21.0%) and $CoCl_2$ (47.2%) and strongly inhibited by $CuSO_4$, $ZnCl_2$, and $HgCl_2$. Hemin, which is an inhibitor of ATP-dependent protein degradation in erythrocytes and an inhibitor of several classes of proteinases (McGuire and DeMartino, 1986), completely inhibited MCP activity at a concentration of 100 μ*M*. The results presented in Table 1 are in agreement with those

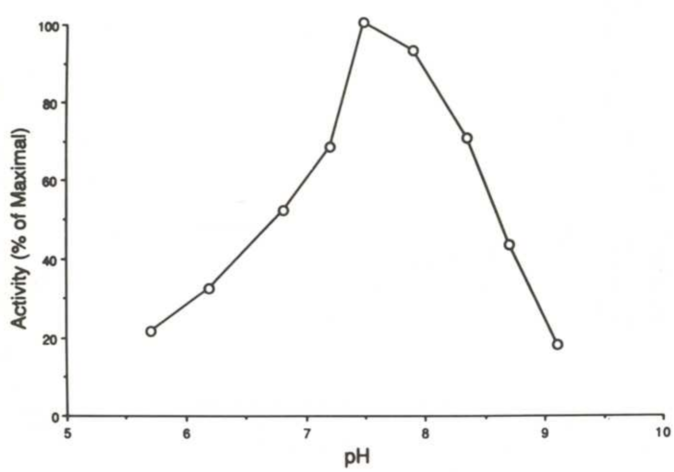

Figure 6. Optimum pH for caseinolytic activity of ovine muscle multicatalytic proteinase complex. The 100-μL aliquots of an enzyme solution (.459 mg/mL) were heated at 60°C for 2 min and an aliquot of the heated fraction was assayed for caseinolytic activity at the indicated pH as described in the Materials and Methods section.

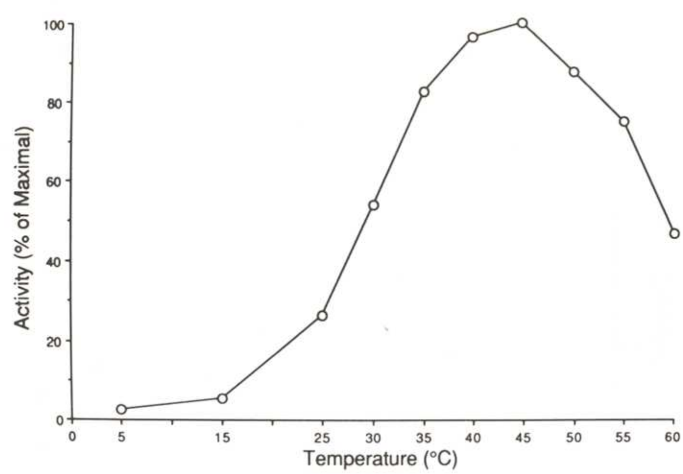

Figure 7. Optimum temperature for caseinolytic activity of ovine muscle multicatalytic proteinase complex. The 100-μL aliquots of an enzyme solution (.459 mg/mL) were heated at 60°C for 2 min, and an aliquot of the heated fraction was assayed for caseinolytic activity at the indicated temperature as described in the Materials and Methods section.

Table 2. Comparison between caseinolytic activities of ovine skeletal muscle multicatalytic proteinase complex (MCP), μ-calpain, and m-calpain[a]

Proteinase	Specific activity, mg of casein degraded/mg of protein
MCP	2.0 ± .05
μ-calpain	44.0 ± .42
m-calpain	59.7 ± 1.53

[a]For details see Materials and Methods section.

reported by other laboratories for MCP from several sources (Dahlmann et al., 1983, 1985a; McGuire and DeMartino, 1986; Tanaka et al., 1986a; Zolfaghari et al., 1987; Mykles, 1989a,b). Because of the multicatalytic nature of MCP, results different than those reported could be obtained, depending on the substrate used. For example, leupeptin selectively inhibited the trypsin-like activity but had no effect on the peptidylglutamyl-peptide activity (Orlowski, 1990).

There are four recognized classes of known proteinases, termed serine, cysteine, metallo-, and aspartate (Kay, 1982). The basis for these classifications is the essential amino acid residue for the catalysis in the active site of the proteinase. Because of the unusual proteinase inhibitor profile indicated for ovine skeletal muscle MCP in Table 1, the proteinase has been considered to have multiple catalytic activities (DeMartino et al., 1991). These activities include trypsin-like (cleavage on the carboxyl side of basic residues), chymotrysin-like (cleavage on the carboxyl side of hydrophobic residues), and peptidylglutamyl-peptide hydrolyzing (cleavage of the carboxyl side of the glutamyl residues) activity (Orlowski, 1990). Examination of the effect of SDS on proteolysis has indicated that the main component responsible for degradation of protein is the peptidyl-

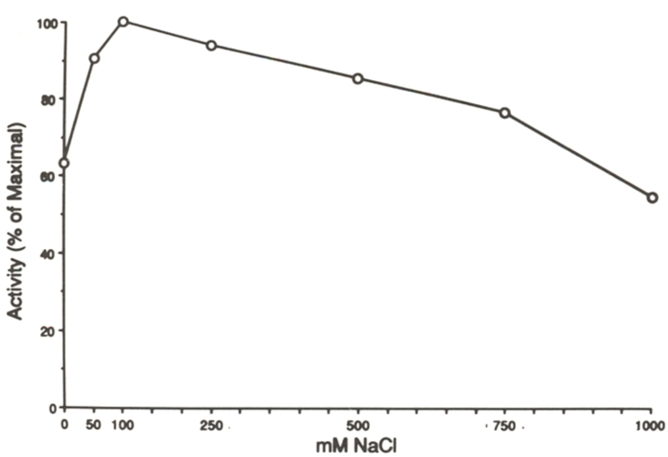

Figure 8. Effect of ionic strength on the caseinolytic activity of ovine muscle multicatalytic proteinase complex. The 100-μL aliquots of an enzyme solution (.459 mg/mL) were heated at 60°C for 2 min and an aliquot of the heated fraction was assayed for caseinolytic activity at the indicated NaCl concentration as described in the Materials and Methods section.

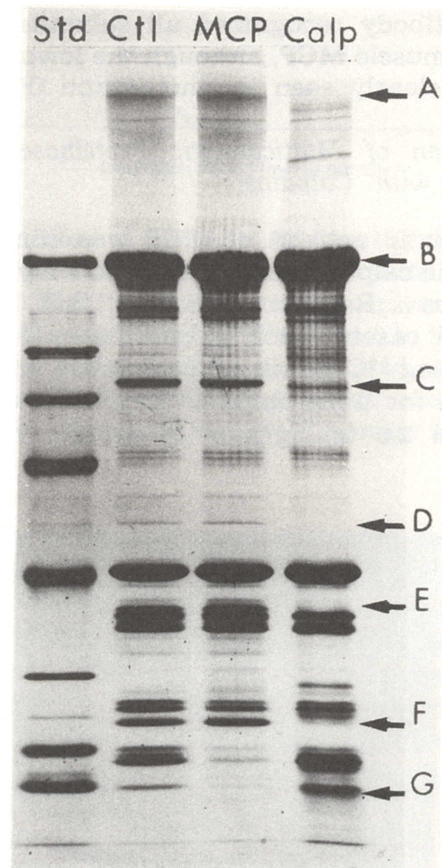

Figure 9. The SDS-PAGE analysis (7.5 to 15% gradient gel) of the effects of muscle multicatalytic proteinase complex (MCP) and μ-calpain (Calp) on ovine skeletal muscle myofibrils. Seventy micrograms of myofibrillar proteins was electrophoresed under denaturing conditions on 7.5 to 15% gradient gels. The first lane is the molecular weight standards (Std) consisting of myosin (200 kDa), *E. coli* β-galactosidase (116.25 kDa), rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa). The second lane is unincubated control (Ctl; myofibrils that were incubated at 45°C for 4 h or incubated with EDTA at 25°C for 4 h had the exact same profile). The third and fourth lanes are myofibrils incubated with MCP and Calp. Arrows A, B, C, D, E, F, and G correspond to the position of titin, myosin heavy chain, alpha-actinin, desmin, troponin-T, troponin-I, and myosin light chain-3, respectively.

glutamyl-peptide hydrolyzing activity (Orlowski and Michaud, 1989).

The results of the present study indicate that ovine skeletal muscle MCP is biochemically similar to MCP isolated from other mammalian and nonmammalian tissues. These results were further substantiated when antibody raised against human erythrocytes (McGuire et al., 1988) cross-reacted with ovine skeletal muscle MCP. The anti-MCP antibody recognized all subunits of ovine skeletal muscle MCP, although the lower subunits are not clearly seen in photograph (Figure 1E).

Comparison of Multicatalytic Proteinase Complex with Calpains

Proteolytic activity of MCP was compared to that of the calpains using casein and myofibrils as substrates. Results indicate that calpains degraded casein much more efficiently than did MCP. Using ^{14}C-casein as a substrate, the specific activities for μ-, m-calpain, and MCP were 44.0, 59.7, and 2.0 (milligrams of protein degraded/milligrams of proteinase), respectively (Table 2). Because μ- and m-calpain have similar effects on myofibrils, only μ-calpain was used to compare the effects of the calpains and MCP on myofibrils (Figures 9, 10, 11, and 12). Classical effects of calpains, which include degradation of Z-disks, titin, desmin, troponin-T, and troponin-I and removal of α-actinin, were observed (Figures 9, 10, and 12). However, myofibrils were a very poor substrate for MCP. Of all myofibrillar proteins, only troponin-C and myosin light chains-2 and -3 were degraded by MCP. Based on phase and electron microscopy observations, MCP had no detectable effect on myofibrils (Figures 10 and 11).

Discussion

The accumulation of muscle tissue or muscle growth depends on the balance between muscle protein synthesis and muscle protein degradation. Therefore, to manipulate muscle growth, knowl-

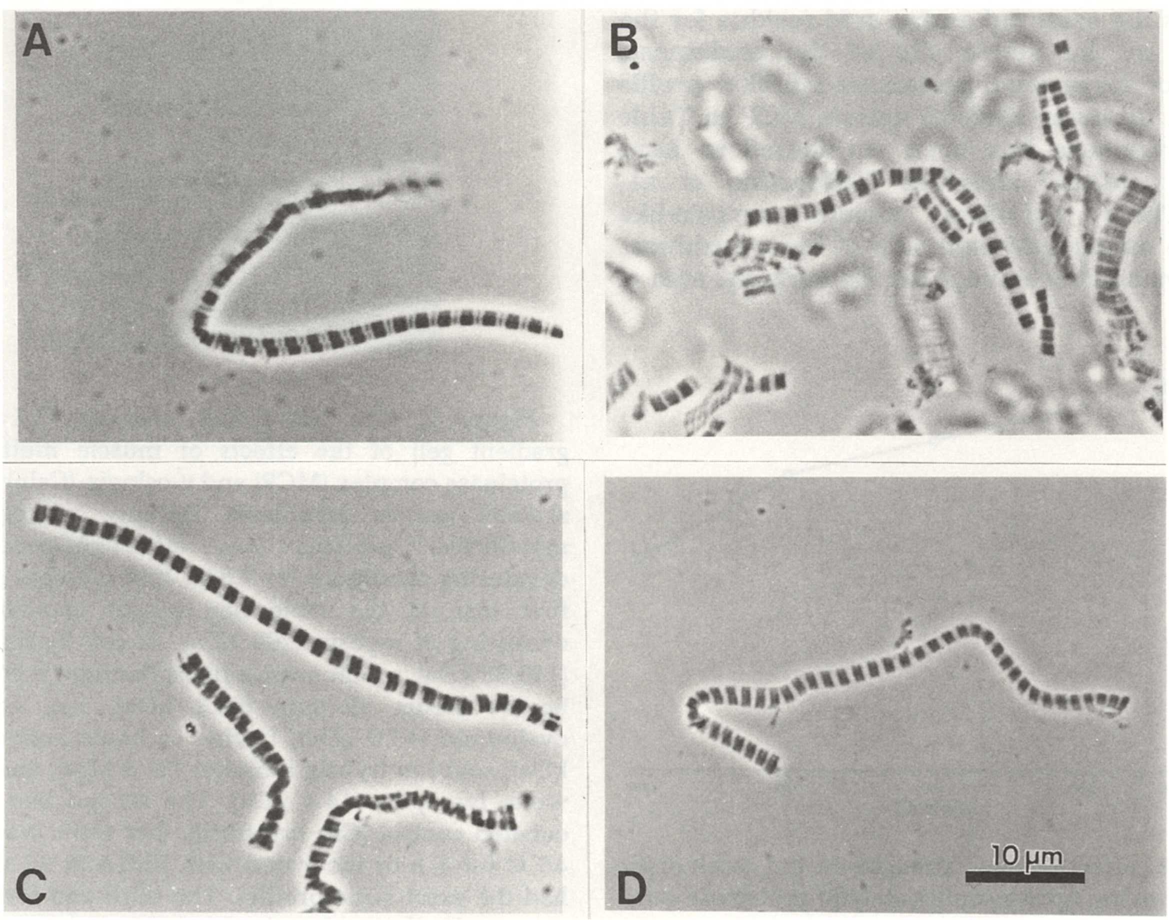

Figure 10. Phase micrograph of myofibrils prepared from controls incubated with μ-calpain in buffer that contained 10 m*M* EDTA for 4 h at 25°C (A), the same as A but that contained 3 m*M* $CaCl_2$ (B), from control incubated with buffer that contained .25 m*M* SDS for 4 h at 45°C (C), and same as B but that contained muscle multicatalytic proteinase complex (D).

edge of both processes is essential. Although much is known about the mechanisms of protein synthesis, very little is known about protein degradation. It has been hypothesized that at least three proteolytic systems could be involved: 1) the lysosomal cathepsins, 2) the calpains, and 3) the MCP (Goll et al., 1989). Although the potential degradative capacity of lysosomes would be sufficient for both basal and induced intracellular protein degradation (Dahlmann et al., 1983), a substantial amount of experimental evidence has been accumulated to suggest that lysosomal enzymes are not involved in the initiation of myofibrillar protein degradation in healthy muscle cells (Goll et al., 1989). In skeletal muscle, because the process of myofibrillar protein breakdown occurs in the cytosol, the proteolytic systems involved must be neutral proteinases. These include the calpains and the MCP. Although the effects of calpains on the myofibrillar proteins are

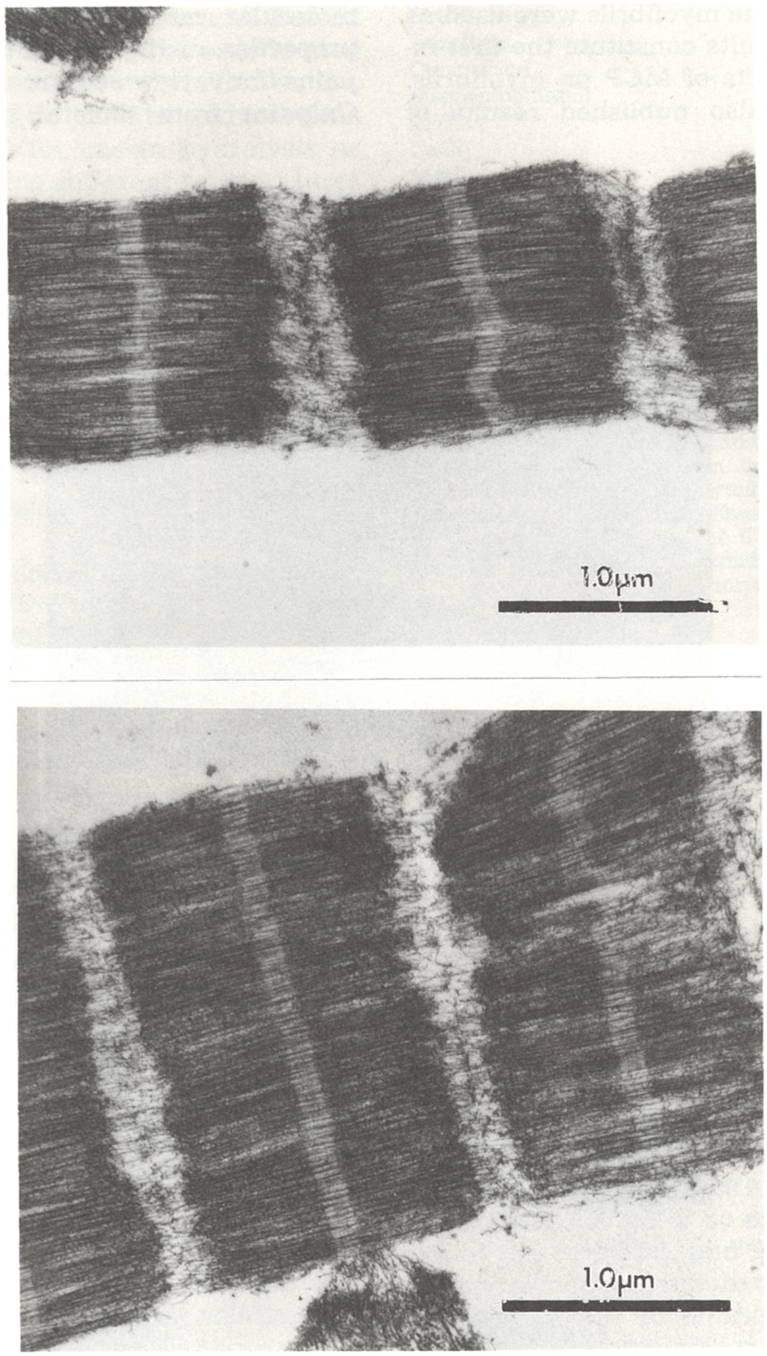

Figure 11. Electron micrograph of myofibrils incubated with buffer that contained .25 mM SDS at 45°C for 4 h (top) or incubated with buffer that contained .25 mM SDS and muscle multicatalytic proteinase complex at 45°C for 4 h (bottom).

well documented and form the basis for the hypothesis that calpains initiate disassembly of myofibril (reviewed by Goll et al., 1989, 1992), very little is known about the effects of MCP on myofibrils.

Ovine skeletal muscle contains a high-molecular-weight proteinase that resembles the MCP common to other mammalian and nonmammalian tissues (Rivett, 1989; Orlowski, 1990). Ovine skeletal muscle β-calpain and m-calpain have 22- and 30-fold greater caseinolytic activity than does MCP from the same tissue. Similar results were obtained when ovine myofibrils were used as the substrate. These results constitute the first in-depth study of the effects of MCP on myofibrils. Mykles (1989a,b) has also published results of incubation of myofibrils with MCP and a calcium-dependent protease from lobster. Mykles (1989b) reported that lobster calcium-dependent protease degraded troponin-I, troponin-C, actin, myosin heavy chain, and myosin light chain-1 and -2. Although these results differ than those reported in the present study, both the myofibrils and MCP in Mykles' study were isolated from lobster muscle and available evidence indicates the calcium-dependent protease isolated from lobster is different from calpains. For example, lobster muscle contains four calcium-dependent proteases with molecular masses of 59 to 310 kDa and with properties distinctly different from those of calpains (for review see Croall and DeMartino, 1991). Calpains from skeletal muscle do not degrade

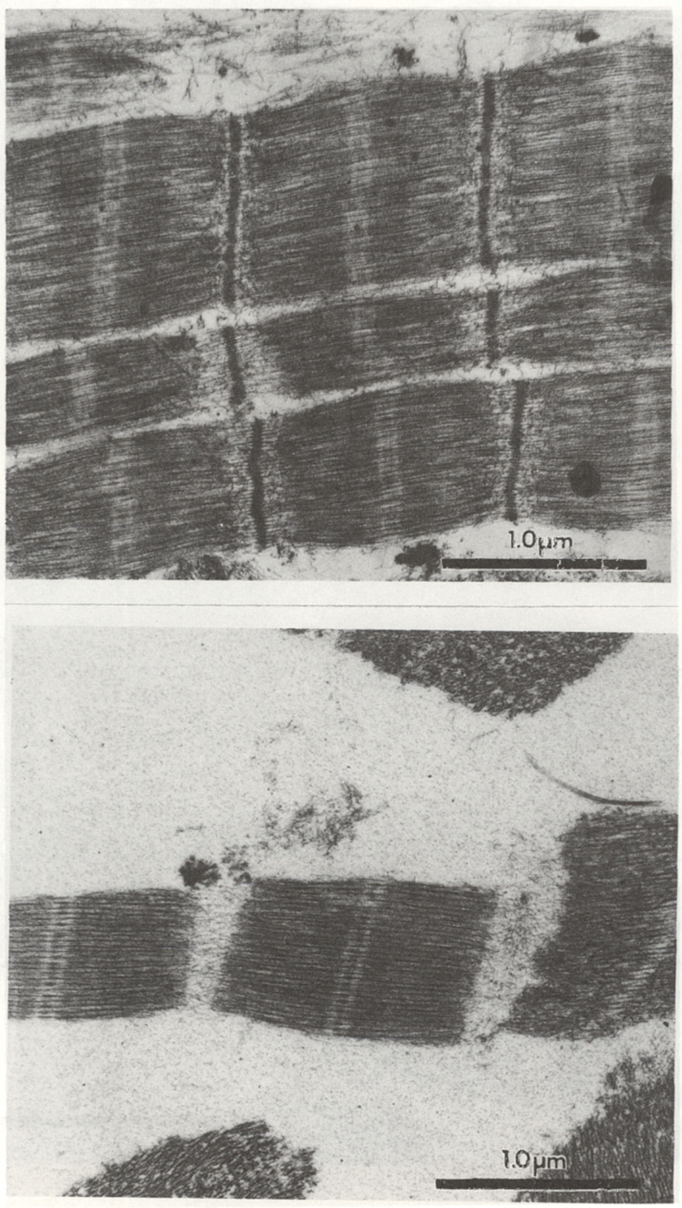

Figure 12. Electron micrograph of myofibrils incubated with μ-calpain with 10 mM EDTA (top) or 3 mM $CaCl_2$ at 25°C for 4 h (bottom).

actin or myosin heavy chain (Goll et al., 1992; Koohmaraie, 1992b). Therefore, it is doubtful if the proteases isolated from lobster (Mykles, 1989a,b) are calpains.

In agreement with the results of the present study, Mykles (1989b) reported that myofibrillar proteins were poor substrates for lobster MCP. Results of our study indicate that of all myofibrillar proteins, MCP degrades only troponin-C, and myosin light chains-2 and -3 were degraded by MCP. More importantly, MCP had no detectable effects of the morphology of myofibrils. Although Mykles (1989a,b) did not examine the effects of MCP on the morphology of myofibrils, he reported that lobster MCP degraded myosin light chains, troponin-C, and myosin heavy chain.

According to Goll et al. (1989, 1992), the initial step of myofibril disassembly involves the release of myofilaments from the surface of myofibril. Release of myofilaments from myofibril would require that the Z-disk, which anchors the thin filaments to the myofibril, and titin and nebulin, which anchor the thin filaments and thick filaments, be released (Goll et al., 1992). Although incubation of myofibrils with calpains resulted in all of these changes (Figures 9, 10, and 12), MCP did not induce any of these changes. Therefore, it is doubtful if MCP is involved in the initial steps of myofibril disassembly.

After the initial myofibril disassembly, the released filaments could either reassociate with its parent or another myofibril or it could be degraded into free amino acids by one or more cytosolic proteases and eventually by lysosomal cathepsins (Goll et al., 1992). At present, it is not known whether the initial step of myofilament release from myofibrils is the rate-limiting step in the myofibrillar protein turnover. Also, it is not known whether MCP participates in further degradation of myofilaments.

Implications

Ovine skeletal muscle contains the multicatalytic proteinase complex. The ovine complex is similar in size, subunit composition, and biochemical characteristics to that in other mammalian and nonmammalian tissues. The proteolytic capacity of multicatalytic proteinase complex was much less than that of the calpains, and it is unlikely that it is involved in the turnover of myofibrils. Also, it can be concluded that multicatalytic proteinase complex does not have a direct role in the postmortem proteolysis of myofibrils. However, it may have an indirect role by acting as a regulator of the proteolytic system involved in the postmortem protein degradation.

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